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Cytocentrin is a Ral-binding protein involved in the assembly and function of the mitotic apparatus

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SUMMARY

Cytocentrin is a cytosolic protein that transiently associates with the mitotic spindle poles in early prophase, and dissociates from them after completion of mitosis. Cloning of its cDNA demonstrated a high degree of homology with three proteins known to specifically interact with an activated form of Ral. Herein we demonstrate that overexpression of cytocentrin inhibits assembly of the mitotic spindle without affecting polymerization or distribution of interphase microtubules. Conversely, loss of cytocentrin expression leads to formation of monopolar spindles. These results indicate that association of cytocentrin with the centrosome may be essential for a timely separation of the diplosomes. They also implicate Ral GTPases and their related pathways in the assembly and function of the mitotic apparatus.

Key words: Cytocentrin, Mitotic Pole, Centrosomal Cycle, Ral

INTRODUCTION

The importance of the centrosome in cellular physiology is well established, but many aspects of its structure, composition and function remain elusive. During interphase, it controls the number, polarity and arrangement of cytoplasmic microtubules, influencing the cellular distribution of organelles such as the ER and the Golgi complex, cell polarity, and the overall three-dimensional shape of cells; during mitosis centrosomes play a key role in the generation of the bipolar spindle (reviewed by Glover et al., 1993). Ultrastructural examination has revealed a diversity of morphological components, including a mother-daughter pair of centrioles (not always present), a convergent set of microtubules, and a variety of electron-dense structures known as pericentriolar material (PCM) (Brinkley, 1985; Rieder and Borisy, 1982). How the different activities of the centrosome are regulated during the cell cycle, and in particular the transition between interphase and mitotic centrosomes, is still largely unknown but, through a combination of immunological and genetic techniques, several of its components have been identified. Genetic approaches led to the discovery of the γ-tubulin gene in *Aspergillus nidulans* (Oakley and Oakley, 1989); this protein is specifically localized in the PCM and all available evidence suggests that it plays a role in microtubule nucleation throughout the mammalian cell cycle (Joshi et al., 1992). A variety of other proteins, including some that display a cell cycle-dependent centrosomal localization, have been discovered using monoclonal or polyclonal antibodies, in many cases obtained from autoimmune disease patients. Prominent among them are NuMA (Price and Pettijohn, 1986), located in the nucleus of interphase cells and redistributing to the spindle poles during mitosis, and cenexin, a marker associated only with the 'mature' centriole (Lange and Gull, 1995). From a functional point of view, NuMA is known to participate in the assembly of the mitotic spindle together with cytoplasmic dynein (Merdes at al., 1996), and Centrin plays an essential role in the duplication of the centrosomes during the cell cycle (Salisbury, 1995). Several motor proteins belonging to the kinesin family have been associated with the mitotic apparatus and the kinetochores, and have been implicated in chromosome movement, in the sliding of microtubules and in the separation of the asters (reviewed by Walczak and Nmitchison, 1996).

Using the mAb RK7, produced against rat cytoskeletal components (Quaroni et al., 1991), we have identified cytocentrin as a protein whose salient feature is its ability to cycle between the cytosol and the mitotic poles, thus potentially representing a regulator of centrosomal functions related to mitosis (Paul and Quaroni, 1993). Taken together, its localization and behavior in cells treated with drugs that alter microtubular functions (nocodazole and taxol), and its apparent inability to interact with interphase microtubules, appear to be inconsistent with a function in the nucleation of the spindle fibers or their interaction with chromosomes. This was particularly evident in mitotic cells recovering from nocodazole treatment, where many of the newly forming nucleating centers lacked significant amounts of associated cytocentrin.

To further investigate the structure and function of cytocentrin we have used mAb RK7 to clone its cDNA. The results presented here indicate that cytocentrin is a Ral-binding

protein involved in the regulation of diplosome separation and assembly of the mitotic spindle.

MATERIALS AND METHODS

Antibodies

The mAbs RK7, recognizing rat cytocentrin (Paul and Quaroni, 1993), and KMX-1, to β-tubulin (Birkett et al., 1985), were described previously. A rabbit antiserum to human lamin B1 was obtained from Drs G. Blobel and N. Chaudhary (The Rockefeller University, New York, NY). The rabbit antibody to γ-tubulin was from Sigma (Product No. T3559). A chicken-anti-cytocentrin antiserum was prepared by injecting His-tagged cytocentrin produced in *E. coli*, denatured in 1% SDS, into the breast muscle of White Leghorn chickens. Initial screening revealed strong reactivity to the His tag, thus specific anti-cytocentrin antibodies were obtained by sequential affinity purifications: (1) cytocentrin cDNA fragments spanning nucleotides 120-1369, 1361-2309 and 2469-3461 were subcloned into pTrcHis, and the corresponding bacterially produced proteins containing the amino-terminal, central (including the RK7 epitope) and carboxy-terminal segments, respectively, were coupled to Affigel 10; (2) the sera were absorbed with the central and carboxy-terminal cytocentrin segments, and then purified using the amino-terminal segment; (3) the His tag was removed by low pH cleavage (incubation with 70% formic acid + 6 M guanidine for 3 days), and the resulting cytocentrin fragment was blotted onto a nitrocellulose membrane and used for a final affinity purification. These antibodies were found to recognize rat, human and monkey cytocentrin on western blots, but did not work for immunofluorescence studies.

Cell culture

Cells were obtained from the American Type Culture Collection (Rockville, MD). In transfection experiments employing glucocorticoid-inducible vectors (pGRE5 or PGRE5/EBV) they were cultured with heat-inactivated and charcoal-extracted FBS. Dexamethasone was used at 100 nM final concentration.

Immunofluorescence microscopy, protein analysis and immunoblotting

Immunofluorescence staining, protein analysis and western blotting were performed as described previously (Paul and Quaroni, 1993). In triple-labelling experiments DNA was stained with DAPI (4′,6-diamidino-2-phenylindole).

DNA cloning, sequencing and analysis

Cloning of the cytocentrin cDNA: poly(A)$^+$ mRNA purified from IEC-17 cells with Promega PolyA-Tract System III (Promega, Madison, WI) was used to construct a unidirectional cDNA library with the ZAP-cDNA Synthesis kit from Stratagene (La Jolla, CA). It was screened by immunoblotting with the RK7 mAb, and from approximately 9×10^5 plaques five strongly positive clones were derived. The corresponding phagemids (designated pBSCC3, pBSCC4, pBSCC5, pBSCC8, pBSCC47) were analyzed by restriction mapping and sequencing. The results indicated that all five clones were derived from the same mRNA. This was confirmed by in vitro synthesis and SDS-PAGE analysis of the polypeptides they coded for using the TnT Coupled Reticulocyte Lysate System from Promega. The 5′ terminal segment missing from the longest insert (pBSCC47) was synthesized by anchored PCR using a 5′ RACE kit from GIBCO-BRL (Gaithersburg, MD): two nested antisense PCR primers (PCR3, TCCACTGTTTAACAACATCGGCGGC and PCR4, GTACATCAT GGTCCTCTCTACTGCG) were placed downstream of a unique *Xma*III site (located at nucleotide 583 of the full-length cytocentrin cDNA). This site was used to insert the segment synthesized by PCR into *Xma*III-cut pBSCC47, and generate the full-length cytocentrin cDNA (pBSCYTO). Sequencing of the cDNA was primarily by the chain-termination method using an Applied Biosystems 373A Automated Sequencer (Foster City, CA); manual sequencing, employing a Sequenase 2.0 kit from USB or a Cyclist Exo-Pfu DNA sequencing kit from Stratagene, was used to overcome ambiguities; standard dideoxynucleotide termination reactions containing 7-deaza dGTP were used to resolve severe GC band compressions. Each region of pBSCYTO was independently sequenced at least 3-4 times.

RNA preparation and northern analysis

Total RNA was extracted from rat tissues and from cell lines as described previously (Paul and Quaroni, 1993), denatured with formaldehyde (6.6%), and resolved by electrophoresis on 1.2% agarose gels, then electroblotted onto Tropilon Plus membrane (Tropix, Bedford, MA) and crosslinked by u.v. irradiation (Stratalinker, Stratagene). The DNA probes were for cytocentrin, a *Pst*I fragment spanning nucleotides 655-1369; for keratin 8, a 505 bp *Bam*HI-*Eco*RI fragment derived from pRK8 (Calnek and Quaroni, 1993); for actin, a mouse β-actin 1.3 kb insert cut with *Xho*I and *Eco*RI from the plasmid pBSmBA obtained from Stratagene. The probes were biotinylated using a Non-Radioactive Random Octamer Labeling System from Tropix. Pre-hybridizations (1 hour) and hybridizations (10 hours) were at 68°C in 1 mM EDTA, 250 mM sodium phosphate, pH 7.2, 5% dextran sulfate, 7% SDS. Biotin-labelled probes (400 ng mixed with 2 mg denatured salmon sperm DNA) were boiled for 10 minutes and cooled on ice before adding to the hybridization buffer. The membranes were washed sequentially at room temperature with 2×SSC + 1% SDS, at 65°C with 0.1×SSC + 1% SDS, at room temperature with 1×SSC. Subsequent blocking (I-Block reagent), incubation with AVIDx-AP and chemiluminescent substrate (CSPD) were performed as recommended by the Southern-Light Detection System (Tropix). The blots were exposed to Kodak XAR films for 2 minutes to 6 hours.

Plasmid constructs

For full-length cytocentrin expression

The 5′ terminal region of cytocentrin extending to the unique *Xma*III site (nucleotides 1-583) was replaced with a PCR-generated fragment spanning nucleotides 120-583 containing an artificial *Bam*HI site adjacent to the ATG start codon. This construct, amplified in pBluescript SK(−) [=pBSCCB10B] and verified by sequencing, was subsequently subcloned into pEBVHisA (Invitrogen, San Diego, CA) [=pB10BEBVA], pcDNA3 (Invitrogen) [=pcDNA3B10B] and pGRE5-1/EBV (Mader and White, 1993) [=pGRE5CCB10B]; for expression in *E. coli* and purification of 6×His-tagged cytocentrin it was also subcloned into pTrcHisA (Invitrogen) [=pB10BTrcA].

For antisense experiments

Three cytocentrin segments were subcloned into pGRE5-1/EBV or pGRE5-2/EBV vectors in either sense or antisense orientation: (1) a segment comprising nucleotides 120-1361(*Bam*HI-*Bgl*II segment from pBSCCB10B) [=pGRE5/EBV1-BamBgl (sense) and pGRE5/EBV2-BamBgl (antisense)]; (2) a segment comprising nucleotides 1361-3622 (*Bgl*II-*Kpn*I segment from pBSCC4) [=pGRE5/EBV1-BglKpn (antisense) and pGRE5/EBV2-BglKpn (sense)] and (3) a segment comprising nucleotides 1167-2122, synthesized by PCR [=pGRE5/EBV1-Mi (sense) and pGRE5/EBV2-Mi (antisense)].

Expression in bacteria

Full-length cytocentrin with an aminoterminal 6×His tag for purification on ProBond resin (Invitrogen) was obtained by transforming the *E. Coli* TOP10 strain with the pB10BTrcA plasmid. Cytocentrin expression was induced with 100 mM IPTG for 6 hours, and cytocentrin was eluted from ProBond with 200 mM imidazole.

Transient and stable transfections

Plasmids purified by double-banding in CsCl were used in all cases. Transient transfections were by the DEAE-Dextran method using a

ProFection kit from Promega (Madison, WI). Cells (in 35 mm dishes) were incubated with DEAE-Dextran (1 mg/ml in PBS) for 9 minutes at room temperature, washed, and then incubated with plasmid DNA (2 μg/dish in 150 μl PBS) for 30 minutes at 37°C. After addition of 2 ml DME supplemented with 10% NuSerum (Becton Dickinson, Bedford, MA) and 80 μM chloroquine the cells were returned to the incubator for 4 hours at 37°C. Finally, the media were aspirated and the cells were exposed to 10% dimethylsulfoxide in PBS for 1 minute at room temperature; following two washes with PBS, they were incubated with standard complete medium. They were examined by immunofluorescence staining 2 days after transfection.

Stable transfections were performed by the BES/calcium phosphate method (Chen, 1996). Cells (in 60 mm dishes) were seeded 1-2 days before transfection and were 30-50% confluent at the start of the procedure; for each dish 20-30 μg plasmid DNA were added to 250 μl of 0.25 M $CaCl_2$; 250 μl 2× BES-buffered solution were added, mixed, then left for 15 minutes at room temperature; the suspension was added dropwise to each dish containing 4 ml complete medium. The dishes were incubated for 24 hours at 35°C with 3% CO_2. Then the medium was removed, the cells were washed twice with PBS, and refed complete medium. Stable transfectants were selected with hygromycin (320 μg/ml); clones were selected with cloning cylinders and serially expanded.

RESULTS

Cloning, sequence and structural features of cytocentrin

Since the mAb RK7 was found to crossreact with keratin 19 and cytocentrin, as a source of mRNA we have selected the rat intestinal cell line IEC-17, known not to express keratin 19 (Chandler et al., 1991). Screening of a Lambda ZAP library with mAb RK7 produced five clones, all derived from the same mRNA, attesting to the excellent specificity of the antibody. The largest insert obtained (in pBSCC47) spanned nucleotides 360-3622 of the full-length cDNA; the 5′ terminal segment was synthesized by anchored PCR and inserted into pBSCC47 utilizing a unique *Xma*III site.

The complete cytocentrin sequence is available from GenBank under accession number U82623. It contains a single large open reading frame spanning nucleotides 120-2201 and coding for 693 amino acids. The AUG start site is flanked by a good Kozak (Kozak, 1986) consensus sequence (cagcatgact), leading to efficient in vitro synthesis using T3 RNA polymerase in a TnT Coupled Reticulocyte Lysate System. The largest [^{35}S]methionine-labelled polypeptide produced exactly comigrated on SDS-PAGE with cytocentrin affinity purified from L6, IEC17 and MH1C1 cells (data not shown). Three less intensely labelled radioactive bands of apparent molecular mass 73, 70 and 60 kDa were observed, indicating efficient translational initiation also from internal AUG codons. Bacterially synthesized His-tagged cytocentrin also displayed the same mobility on gels as the affinity-purified protein, but it should be noted that the size of cytocentrin deduced from its sequence (80,068 Da) is considerably lower than that calculated from SDS-PAGE gels (102 kDa). This abnormally slow migration was also noted for RalBP1 (Cantor et al., 1995; see below).

Analysis of cytocentrin's amino acid sequence revealed a predominance of predicted α-helical regions (86%) distributed among six major domains, and a large fraction of charged amino acids (42.28%). Asp, Glu and Lys residues are particularly abundant in the second α-helical region, where they represent 62/110 residues. Here, a novel motif consisting of LysGluGluLysHis repeated three times (positions 132-146) was noted, embedded in a remarkably charged α-helical segment. A pattern search against the Prosite database of sequence motifs and patterns revealed potential phosphorylation sites for cAMP-dependent kinases, protein kinase C, casein kinase II and a single tyrosine-phosphorylation site. A leucine zipper and three potential myristylation sites were also noted, but no potential membrane-spanning domains, eukaryotic secretory signal sequences, or signals for import into cytoplasmic organelles. In addition, five 'good' and four 'poor' PEST sequences have been identified: these regions rich in Pro, Glu, Ser, Thr and, to a lesser extent, Asp (usually flanked by positively charged amino acids) have been described in eukaryotic proteins with half lives of less than 2 hours, and are rarely found in intracellular proteins with half lives of more than 20 hours (Rechsteiner, 1990). Thus, their presence in cytocentrin is indicative of a relatively short half life for this protein.

Homology or identity with Ral-binding proteins

Search of the GenBank database identified three cDNAs highly homologous or nearly identical to that of cytocentrin: RalBP1 (Cantor et al., 1995), RLIP76 (Jullien-Flores et al., 1995) and RIP1 (Park and Weinberg, 1995). Their corresponding protein sequences are compared with that of cytocentrin in Fig. 1, where analyses of similarity at the DNA (Fig. 1B) and protein (Fig. 1C) levels and a phylogenetic tree based on their nucleotide sequences (Fig. 1D) are also presented. RalBP1 and RLIP76 have been identified using yeast-based two-hybrid systems to screen rat or human cDNA libraries, respectively, for proteins interacting preferentially with the GTP-bound form of RalA, while RIP1 was cloned by screening a mouse cDNA expression library with a modified radiolabelled version of human RalB. Similarity among them and cytocentrin is higher at the protein sequence level (89.9-97.5%) because of the relatively low homology of their 3′ terminal non-translated regions. RalBP1 was, like cytocentrin, derived from a rat cDNA library, and their sequences are nearly identical, displaying significant diversity (45% identity) only in the first 23 nucleotides; thereafter, there are 12 single nucleotide substitutions in the region spanning nucleotides 24-2484 of cytocentrin. However, the presence of an extra nucleotide (a 'C' at position 2027) in cytocentrin extends its ORF considerably (coding for an extra 46 amino acids, see Fig. 1A). It should also be noted that the published RalBP1 sequence was truncated at a position corresponding to nucleotide 2487 of cytocentrin, preventing a comparison of most of their 3′ non-translated regions and leaving open the possibility that they may represent different splice variants. These differences notwithstanding, the 98.4% identity at the protein sequence level indicates that cytocentrin and RalBP1 either are the same gene product or members of a very closely related family of proteins.

Expression in rat cell lines

By northern blotting, a single mRNA with an estimated size (3700 nucleotides) nearly identical to that of the full-length cDNA was detected in RNA fractions purified from IEC-17, L6 and MH1C1 cells (Fig. 2, lanes 1-3). This band was, however,

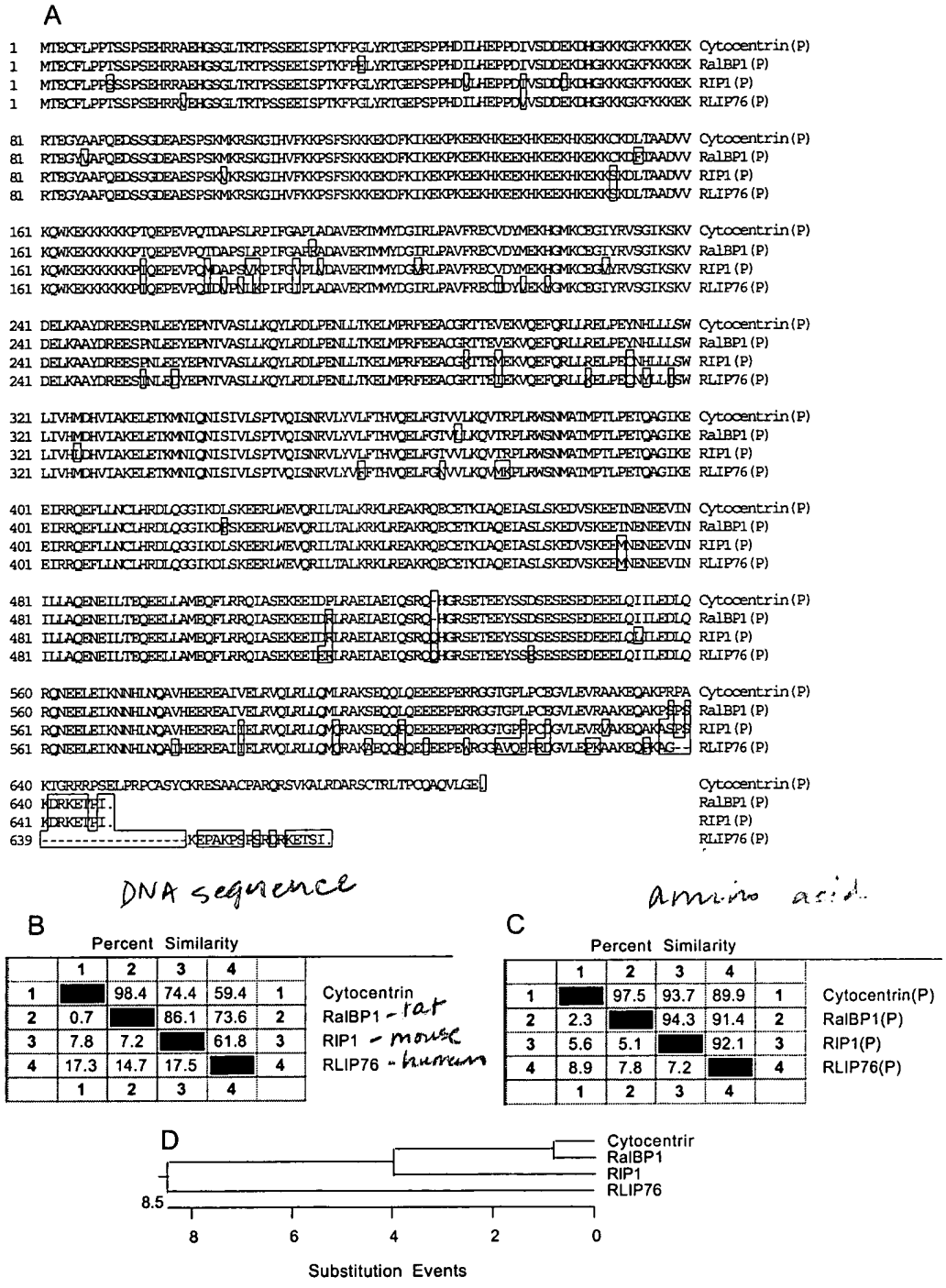

Fig. 1. Homology of cytocentrin with the Ral-binding proteins RalBP1, RIP1 and RLIP76. In (A) are compared the deduced amino acid sequences, and mismatches are highlighted within boxes. The plot of divergence and similarity between each sequence pair, based on the published DNA sequences and calculated using DNAStar Megalign, is presented in (B), while in (C) divergence and similarity were calculated based on the deduced amino acid sequences. A phylogenetic tree based on the Hartigan-Fitch-Sankoff method, as implemented in DNAStar, is presented in (D); it properly reflects the origin of each cDNA (cytocentrin and RalBP1 from the rat, RIP1 from the mouse, and RLIP76 from man).

relatively faint and could only be visualized by prolonged incubation (4-6 hours) with the alkaline phosphatase chemiluminescent substrate. When entire tissues (intestine, liver, kidney) were used for mRNA purification, the same band was barely visible and could not be reproduced photographically (Fig. 2, lanes 4-6). Blot stripping and reprobing for rat keratin 8 (K8 in Fig. 2) or mouse actin (Act in Fig. 2) produced far stronger signals in a much shorter time (2-4 minutes). These results indicate that cytocentrin mRNA is present at very low abundance in all cell types examined, and may be preferentially expressed in proliferative cell populations. This finding is consistent with our previous study (Paul and Quaroni, 1993), where evidence for a very low cellular abundance at the protein level was also obtained by western blotting.

Analysis of function: overexpression

Stable transfection of a variety of rat, monkey and human cell lines (IEC-17, MH1C1, COS-1, COS-7, HeLa) with constructs

containing cytocentrin in three different expression vectors (pEBVHisA, pcDNA3 and pGRE5-1/EBV) consistently failed to produce cells capable of overexpressing cytocentrin. Under selective conditions (G418 or hygromycin), far fewer (5-12%) clones were obtained than when cells were transfected with vectors alone. The absence of any plasmid-encoded cytocentrin mRNA in the few clonal cell lines obtained was verified by RT-PCR and northern blotting. These results indicate that cytocentrin overexpression is incompatible with prolonged cell survival or growth.

High level transient expression of rat cytocentrin in a human or monkey background (recognized by the rat-specific mAb RK7) was obtained in COS-1 and HeLa cells using the pcDNA3- or pGRE5-1/EBV-derived constructs pcDNA3B10B and pGRE5CCB10B.

The major phenotypic alteration observed in these cytocentrin overexpressing cells is shown in Fig. 3. In most mitotic cells (6 of 8 documented by confocal microscopy) the spindles were completely or nearly completely absent (Fig. 3C), and optical sectioning (Fig. 3D-K) showed that the chromosomes were distributed apparently at random over the entire volume of the cell. In the remaining two cells the mitotic apparatus was only partially assembled, and the chromosomes were not properly aligned with respect to the poles (Fig. 3L-N). Based on the RK7 fluorescence intensity, quantified at the confocal microscope as 4.6-fold lower in Fig. 3L than in Fig. 3A, these two cells appeared to express the lowest levels of exogeneous cytocentrin. It is also noteworthy that in these cells rat cytocentrin appeared to associate with the partially assembled spindle components (Fig. 3L), confirming its tendency to bind to microtubular structures but only in mitotic cells, as previously observed in taxol-treated cells (Paul and Quaroni, 1993). Cytocentrin overexpression also had a significant effect on the number and size of the centrosomes present in mitotic cells. As shown in Fig. 4A-C, most RK7-positive cells displayed more than two centrosomes; in addition, they were considerably smaller than centrosomes present in control untransfected cells (Fig. 4E). In contrast with these defects in microtubular structures present in mitotic cells, cytocentrin overexpression had no apparent effects on the morphology of interphase cells and did not significantly alter the frequency or distribution of cytoplasmic microtubules (Fig. 4H). Also, staining for lamin B demonstrated that the nuclear envelope was normally disassembled at mitosis in cytocentrin-overexpressing cells (Fig. 5), suggesting that progression through the cell cycle and other mitotic events were not affected by the presence of plasmid-encoded cytocentrin.

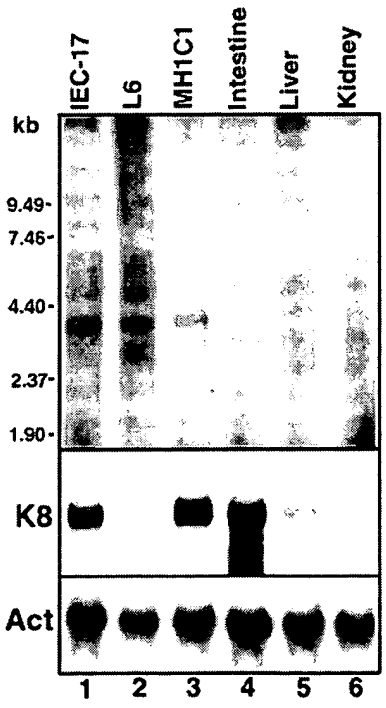

Fig. 2. Northern blot analysis of cytocentrin mRNA expression in rat cell lines and tissues. Purified mRNAs (6 μg/lane) were from IEC-17 (lane 1), L6 (lane 2), MH1C1 (lane 3) cells, and from rat small intestine (lane 4), liver (lane 5) and kidney (lane 6). They were hybridized with a cytocentrin probe, then stripped and re-hybridized with a probe for rat keratin 8 (K8), re-stripped and finally hybridized with an actin probe (Act). All probes were biotin-labelled, and bands were visualized, following incubation with AVIDx-AP, by incubation with an AP chemiluminescent substrate. The blot was exposed to Kodak XAR films for 6 hours (cytocentrin), 4 minutes (keratin 8) or 2 minutes (actin). The RNA standard was a 0.24-9.5 kb ladder.

Analysis of function: antisense experiments

The use of specific antisense RNAs or oligonucleotides to block expression of selected genes has emerged as a powerful tool to investigate the function of the corresponding proteins, but the success rate is unpredictable and dependent on not entirely understood factors (Pestka, 1992). Among them are the level of expression of the antisense mRNA and the necessity for complimentarity with the 5′ end of the gene (Pestka, 1992). We have, therefore, employed various different constructs in this work and, to control expression levels, we have used a dexamethasone-inducible EBV episomal vector containing five high-affinity glucocorticoid response elements (GRE5) placed upstream of the adenovirus 2 late promoter (Mader and White, 1993). Preliminary experiments in transiently transfected COS-1 cells demonstrated very low level expression of rat cytocentrin mRNA in the absence of the inducer and a 20-45 fold increase in the presence of 100 nM dexamethasone. Three different segments of the cytocentrin cDNA were subcloned in either the sense or antisense orientation and used, together with the original vector, in all transfection experiments: a 5′ terminal segment comprising nucleotides 120-1361 [pGRE5/EBV1-BamBgl (sense) and pGRE5/EBV2-BamBgl (antisense)], a segment spanning the mid-third (nucleotides 1167-2122) [pGRE5/EBV1-Mi (sense) and pGRE5/EBV2-Mi (antisense)], and a segment representing the terminal two-thirds of cytocentrin (nucleotides 1361-3622) [pGRE5/EBV2-BglKpn (sense) and pGRE5/EBV1-BglKpn (antisense)]. COS-1 cells were transfected by the BES/calcium phosphate method and clonal cell lines were selected in the presence of hygromycin. The presence of plasmid-encoded rat cytocentrin RNA segments was verified by RT-PCR and, in selected cases, the levels of expression were evaluated by northern blotting. The effectiveness of the antisense constructs in reducing endogeneous cytocentrin expression was verified by western blotting using affinity-purified chicken antibodies specific for the amino-terminal segment (lacking the RK7 epitope) of the bacterially produced rat protein (see Materials and methods for

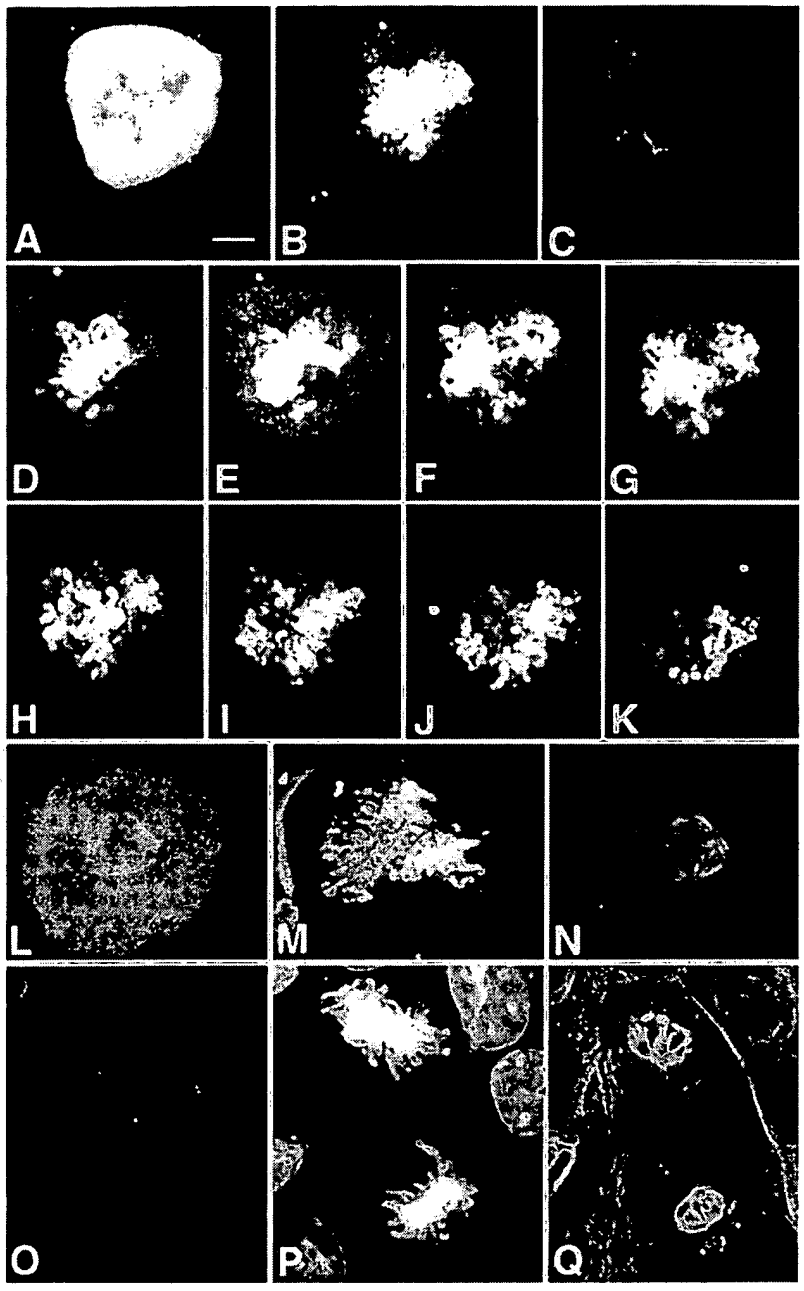

Fig. 3. Transient cytocentrin overexpression inhibits assembly of the mitotic apparatus. COS-1 cells were transfected with the pcDNA3B10B construct, containing the entire rat cytocentrin coding region, by the DEAE-Dextran method. 2 days later the cells were processed for triple-label immunofluorescence staining with the RK7 mAb (specific for rat cytocentrin, not recognizing the monkey homolog, see A,L,O), the KMX-1 mAb (specific for β-tubulin, see C,N,Q), and DNA (B,D-K,M,P). A total of eight overexpressing mitotic cells were documented: six of them had completely or nearly completely absent mitotic apparatus and disorganized chromosomes, as in the cell shown in (A-C). (D-K) Serial 1 μm optical sections, illustrating the presence in this cell of chromosomes apparently randomly distributed over most of the cell volume. Note that these chromosomes appear fragmented because of the thinness of the optical sections. The other two cells displayed lower cytocentrin fluorescence (compare A and L), and a less incomplete, although still abnormal, mitotic apparatus, as shown in (N). A normal, non-transfected, control mitotic cell is shown in (O-Q). Bar, 10 μm.

details). As illustrated in Fig. 6, these antibodies reacted with both rat cytocentrin (affinity-purified from L6 cells with the RK7 mAb, lane 1), and the endogeneous COS-1 homolog displayed nearly identical mobility on SDS-PAGE (lanes 2-11). Only the pGRE5/EBV2-BamBgl construct reduced significantly endogeneous cytocentrin expression (lanes 6,7), to different levels depending on the clone analyzed (the two clones displayed in Fig. 6 represent the maximum and minimum levels detected in five clones examined).

Out of each transfection five clones were selected and examined by immunofluorescence staining with an anti-tubulin mAb. With the exception of pGRE5/EBV2-BamBgl, all other sense and antisense constructs, and the vector alone, produced no detectable alterations in cell morphology, in the distribution of cytoplasmic microtubules or in the organization of the mitotic apparatus. In striking contrast, cells transfected with the 5′ terminal segment of cytocentrin in an antisense orientation (pGRE5/EBV2-BamBgl) displayed a much reduced (3- to 5-fold) growth rate, accumulation of very large cells, and a significant proportion (28-78% in nine separate experiments) of monopolar mitoses (Fig. 7D). Their salient features are illustrated in Fig. 7C-P: optical sectioning (Fig. 7E-L) demonstrated the presence of microtubules radiating from a single pole and entirely surrounded by a sphere of chromosomes. These monopoles appeared very similar or identical to the *mgr* type described by Gonzalez et al. (1988),

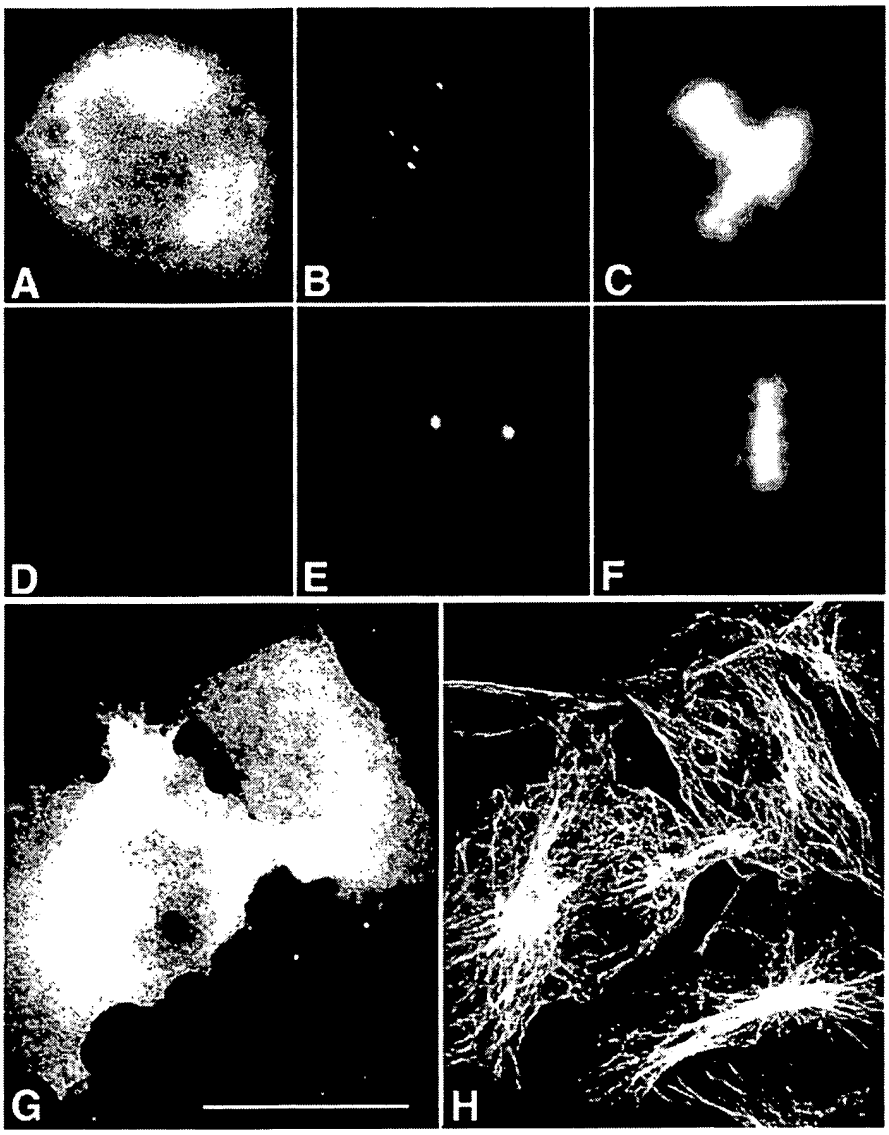

Fig. 4. Staining of centrosomes for γ-tubulin in cytocentrin-overexpressing (A-C) or control (D-F) COS-1 cells. Cells were triple-labelled with the RK7 mAb (A,D), a rabbit antibody to γ-tubulin (B,E) and DAPI (C,F). Note the presence of four centrosomes in the rat-cytocentrin overexpressing mitotic cell (B), much smaller in size than the centrosomes of the untransfected cell (E). (G,H) Interphase COS-1 cells double-labelled for rat cytocentrin (G) and β-tubulin (H), demonstrating that cytocentrin overexpression did not affect assembly of interphase cytoplasmic microtubules. Bar, 25 μm.

and their size was approximately 2-3 times greater than those present in normal bipolar spindles.

Staining for γ-tubulin revealed that in these monopolar mitoses the centrosomes had duplicated but failed to separate (Fig. 7C) as in normal bipolar controls (Fig. 7B). In some cells, microtubular structures still surrounded entirely by chromosomes but containing two distinct asters were also observed (Fig. 7M-P). The overall microtubule mass was much reduced in these miniasters, suggesting that these cells had progressed further in mitosis than cells like that depicted in Fig. 7D. These results indicate that expression of this antisense RNA fragment prevented the timely separation of the diplosomes but, at least in some cases, pole separation eventually could take place from within a 'cocoon' of chromosomes. The presence, in the same cultures, of cells displaying multipolar mitoses, often with up to 8-10 poles/cell, may be attributed to loss or reduced multiplicity of the episomal vector after one or more rounds of abnormal cell growth. Quantitative data, derived from three clones examined for each construct (sense and antisense, plus controls) are presented in Table 1. It should be noted that monopolar mitoses were only observed in cells transfected with the pGRE5/EBV2-BamBgl vector, and their frequency correlated with the relative reduction in endogeneous cytocentrin expression observed by western blotting.

DISCUSSION

Cloning of cytocentrin's cDNA and the discovery of its close homology with proteins RalBP1, RIP1 and RLIP76, independently identified because of their ability to bind to activated forms of Ral GTPases (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995), have provided significant insight into the function of this protein. The homology between cytocentrin and RalBP1 (both of rat origin) is so high (Fig. 1) as to leave little or no doubt that they are products of the same gene. Even if this were not the case, their

Table 1. Relative proportion of monopolar, bipolar and multipolar spindles in COS-1 cells transfected with vector alone (COS/pGRE5-2/EBV) or with the different sense and antisence cytocentrin-derived constructs

Clone	Monopolar	Bipolar	Tripolar	Multipolar	*mgr*-type
COS/pGRE5-2/EBV(1)	3 (3.4%)	77 (87.5%)	7 (7.9%)	1 (1.1%)	0
COS/pGRE5-2/EBV(2)	5 (6.6%)	64 (84.2%)	4 (5.3%)	3 (3.9%)	0
COS/pGRE5-2/EBV(3)	7 (9.0%)	65 (83.3%)	5 (6.4%)	1 (1.3%)	0
COS/EBV1-BamBgl(1)	7 (9.2%)	61 (80.3%)	6 (7.9%)	2 (2.6%)	0
COS/EBV1-BamBgl(2)	3 (4.0%)	58 (77.3%)	12 (16.0%)	2 (2.7%)	0
COS/EBV1-BamBgl(3)	6 (7.3%)	66 (80.5%)	7 (8.5%)	3 (3.6%)	0
COS/EBV2-BamBgl(1)	6 (7.1%)	18 (21.2%)*	5 (5.6%)	9 (10.6%)*	47 (55.3%)*
COS/EBV2-BamBgl(2)	5 (7.5%)	33 (49.2%)*	3 (4.5%)	7 (10.4%)*	19 (28.3%)*
COS/EBV2-BamBgl(3)	7 (8.3%)	21 (25.0%)*	6 (7.1%)	10 (11.9%)*	40 (47.6%)*
COS/EBV1-BglKpn(1)	17 (20.5%)	55 (66.3%)	7 (8.4%)	4 (4.8%)	0
COS/EBV1-BglKpn(2)	9 (9.9%)	72 (79.1%)	6 (6.6%)	4 (4.4%)	0
COS/EBV1-BglKpn(3)	5 (6.7%)	61 (81.3%)	8 (10.7%)	2 (2.7%)	0
COS/EBV2-BglKpn(1)	7 (7.4%)	80 (85.1%)	6 (6.4%)	1 (1.1%)	0
COS/EBV2-BglKpn(2)	5 (6.9%)	57 (79.2%)	7 (9.7%)	3 (4.2%)	0
COS/EBV2-BglKpn(3)	3 (3.7%)	71 (87.6%)	6 (7.4%)	1 (1.2%)	0
COS/EBV1-Mi(1)	5 (7.3%)	52 (76.5%)	9 (13.2%)	2 (2.9%)	0
COS/EBV1-Mi(2)	9 (9.7%)	78 (83.9%)	5 (5.4%)	1 (1.1%)	0
COS/EBV1-Mi(3)	11 (11.3%)	76 (78.3%)	7 (7.2%)	3 (3.1%)	0
COS/EBV2-Mi(1)	10 (11.4%)	71 (80.7%)	6 (6.8%)	1 (1.1%)	0
COS/EBV2-Mi(2)	11 (11.1%)	78 (78.8%)	8 (8.1%)	2 (2.0%)	0
COS/EBV2-Mi(3)	5 (6.9%)	60 (83.3%)	5 (6.9%)	2 (2.8%)	0

For details of the different constructs, see Materials and methods.

Three clones from each transfection were analyzed by double-labelling for tubulin and DNA. Cells were observed by epifluorescence microscopy, and when necessary the presence of *mgr*-type monopolar structures was confirmed by confocal laser imaging.

Monopolar spindles refers to normally occurring structures observed in a variety of cell types (Cassimeris et al., 1994).

Groups statistically different from controls (cells transfected with vector alone) are marked with an asterisk.

amino acid sequences are identical within the Ral-binding region identified in RalBP1, and deletion studies conducted on RalBP1, RIP1 and RLIP76 concur in demonstrating that such a region is both sufficient and necessary for Ral-binding activity. Thus the salient features of cytocentrin, deduced from its sequence and from the studies conducted on its homologs, are a predominantly α-helical conformation and the presence of at least three distinct domains: (1) an amino-terminal region unusually rich in lysines, glutamic and aspartic acids, including continuous stretches of alternating positive and negative charges forming a novel protein motif; (2) a central region of homology with GAP domains that have been involved in the regulation of GTPases of the Rho/Rac family; and (3) a Ral-binding domain in the acidic carboxyl terminus of the protein.

A major conclusion of this study is therefore that a protein, linked through a cascade of GTP-binding proteins and their related effector molecules to pathways activated during mitogenic signaling at the level of the plasma membrane, is a component of the mitotic apparatus and may be implicated in the transition between interphase and mitotic centrosomes. This may also shed new light into the function of Ral GTPases (RalA and RalB), discovered over 10 years ago (Chardin and Tavitian, 1986), whose role in cellular regulation is still unclear. RalA and RalB are similar to Ras in their affinity for guanine nucleotides, and their activity is suppressed by comparable mutations, but have distinct effector domains crucial for their interaction with downstream targets and GAPs (Cantor et al., 1995). It is noteworthy that Ral's localization primarily in cytoplasmic membranes and vesicles (Hofer et al., 1994; Kikuchi et al., 1994; Spaargaren and Bischoff, 1994) appears to coincide with that of cytocentrin in interphase cells (Paul and Quaroni, 1993). This raises the possibility, presently under investigation, that binding to activated Ral may be what prevents association of cytocentrin with the centrosomes until late in G_2 or early prophase. In view of the lower than expected activity and specificity of the RhoGAP domain of cytocentrin's RIP1 homologue, Park and Weinberg (1995) suggested that its true target had yet to be identified. Further, these authors speculated that Ral may serve to recruit RIP1 to a compartment in which it can function or, alternatively, to sequester RIP1, removing it from a compartment in which it can activate effector molecules similar to but not identical with CDC42 or Rac. Our results are consistent with such a model and indicate that at least one important target is a kinase located in the centrosome, where it may be activated through interaction with cytocentrin or cytocentrin/Ral complexes in preparation for mitosis. A protein (RalGDS) linking Ras to Ral has been identified (Albright et al., 1993); thus the entire pathway Ras→RalGDS→Ral→Cytocentrin→Kinase(?) would provide a link with mitogenic regulators implicated in membrane and cytoskeletal modifications that are part of the Ras and Rho pathways. Activation of Ras, and consequently of Ral, could lead to release of cytocentrin from cytoplasmic stores. Among the kinases already known to locate in the mitotic poles and representing candidates for interaction with activated cytocentrin are Aurora, whose deletion leads to formation of unipolar mitoses very similar or identical to those observed in our antisense experiments (Glover et al., 1995), and Plk1

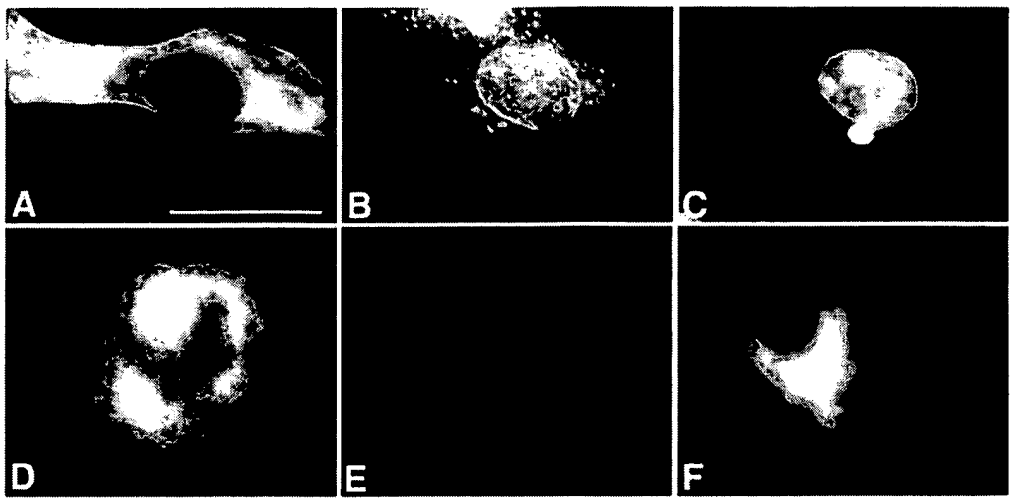

Fig. 5. The nuclear envelope is normally disassembled at mitosis in COS-1 cells expressing rat cytocentrin. Cells transfected with pcDNA3B10B were triple-labelled with RK7 (A,D), a rabbit antiserum specific for lamin B (B,E) and DAPI (C,F). (A-C) An interphase cytocentrin-positive cell displaying normal staining of the nuclear envelope; (D-F) a mitotic cell expressing high levels of rat cytocentrin, with a diffuse cytoplasmic staining for lamin B. Bar, 25 μm.

(Polo-like kinase), whose cellular distribution during interphase and mitosis bears considerable resemblance with that of cytocentrin (Golsteyn et al., 1995). Our working hypothesis could also provide a lead into one of the central issues in the centrosomal cycle, coordination with chromosomal DNA replication to ensure a proper order of events and accurate centrosomal duplication only once during the cell cycle (Bailly and Bornens, 1992). Assuming that cytocentrin is only required for centrosomal function(s) starting in late G_2/early mitosis (i.e. when we have observed it to bind first to the centrosome), and that the main function of cytocentrin is to promote separation of the already duplicated centrosomes, the role of Ras (and its downstream effector molecules) in the centrosome cycle would be to regulate the timing of such a separation. But alternative or complementary function(s) cannot be excluded. For instance, it has been suggested that coordination of centrosome reproduction with nuclear events of the cell cycle may depend on 'licensing' of the centrioles/centrosomes (Hinchcliffe et al., 1998). Phosphorylation of resident centrosomal proteins by a cytocentrin-dependent kinase activated only once during the cell cycle (in late G_2/early mitosis) could represent such a licensing event, manifesting itself during the subsequent S phase.

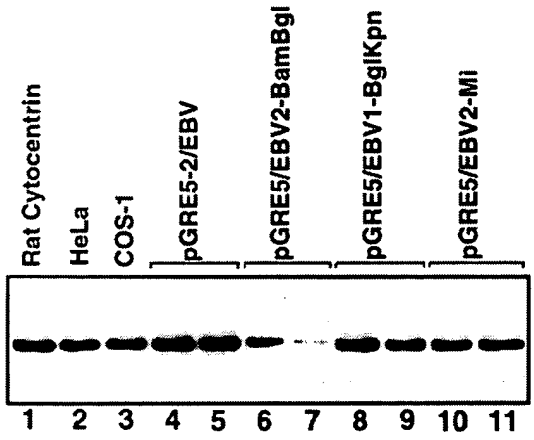

Fig. 6. Endogeneous cytocentrin levels in COS-1 cells transfected with different antisense constructs. Total cell lysates (300 μg protein) were applied to each lane, separated by SDS-PAGE, blotted onto Hybond ECL nitrocellulose membrane, and incubated with affinity-purified chicken-anti-cytocentrin antibodies specific for the amino-terminal portion of the protein (see Materials and methods). The ECL Western Blotting kit from Amersham was used for detection and development: exposure to Hyperfilm-ECL film was for 30 seconds. Control rat cytocentrin, affinity-purified from L6 cells with RK7, was applied to lane 1, and untransfected HeLa or COS-1 cell lysates to lanes 2 and 3, respectively. Two independently derived clones from each transfection are included in this figure; they represent COS-1 cells transfected with vector alone (pGRE5-2/EBV, lanes 4,5), pGRE5/EBV2-BamBgl (nucleotides 120-1361 in antisense orientation, lanes 6,7), pGRE5/EBV1-BglKpn (nucleotides 1361-3622 in antisense orientation, lanes 8,9) and pGRE5/EBV2-Mi (nucleotides 1167-2122 in antisense orientation, lanes 10,11). Note that only pGRE5/EBV2-BamBgl reduced the endogeneous levels of cytocentrin, whose relative amounts correlated with the frequency of monopolar mitoses observed by immunofluorescence staining; the samples in lanes 6 and 7 were from clones COS/EBV2-BamBgl(2) and COS/EBV2-BamBgl(1), respectively, listed in Table 1.

The model discussed above provides a conceptual framework for the interpretation of our studies aimed at investigating cytocentrin's function through its forced overexpression and underexpression (antisense). Underscoring the importance of cytocentrin's normally low cellular concentration, forced overexpression proved incompatible with prolonged cell survival or growth. The likely reason, as demonstrated by the transient transfection experiments, is that the transfected cells could not assemble a complete mitotic apparatus (Fig. 3). However, in overexpressing cells interphase microtubules did not appear affected (Fig. 4), and these cells progressed normally through the cell cycle until the beginning of mitosis, with a timely breakdown of the nuclear envelope (Fig. 5). We have previously observed (Paul and Quaroni, 1993) that, while cytocentrin was never found associated with interphase microtubules, it did appear to bind to the (−) end of some microtubules in taxol-treated cells. Staining of incomplete mitotic spindle components was also observed in overexpressing cells in this study (Fig. 3L). Thus, a plausible explanation is that cytocentrin has the ability to bind to microtubular structures, but with relatively low affinity and only when activated and/or

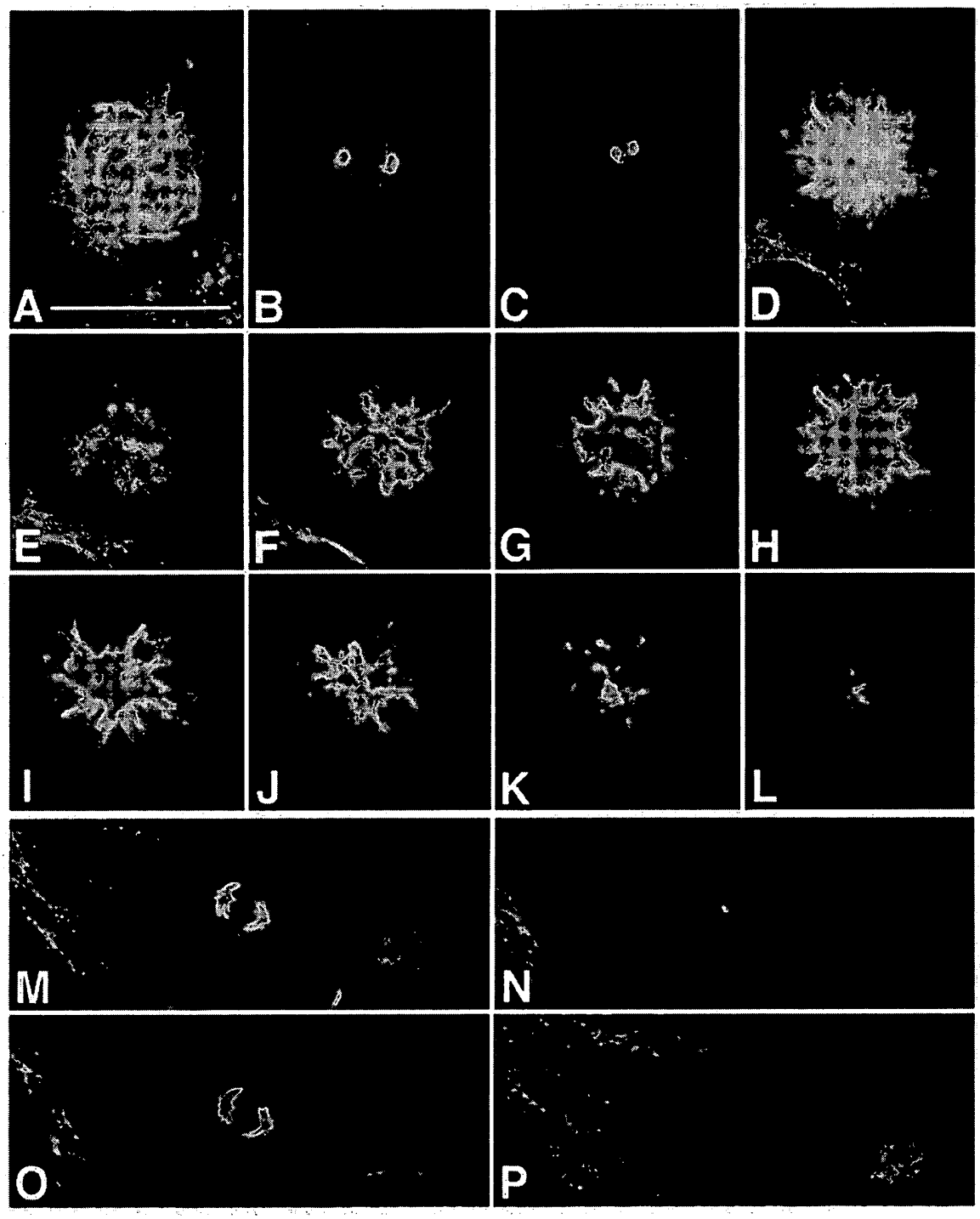

Fig. 7. Formation of monopolar mitoses in COS-1 cells expressing an antisense cytocentrin RNA fragment. Cells were transfected with the plasmid pGRE5/EBV2-BamBgl, containing a 5′ terminal segment of cytocentrin spanning nucleotides 120-1361 in an antisense orientation. Stable transfectants were selected with hygromycin, and individual clones picked with cloning cylinders were serially expanded. Cells double-labelled for β-tubulin (mAb KMX-1, yellow-green fluorescence) (A,D-P), γ-tubulin (yellow-green fluorescence) (B,C), and for DNA with PI (red fluorescence) were examined by scanning confocal microscopy. (A-D) Projections of entire sets of optical sections. (A) A cell transfected with the vector alone, displaying a normal bipolar mitotic apparatus; (D) a monopolar mitosis observed in pGRE5/EBV2-BamBgl-transfected cells, with its individual 1.0 μm optical sections presented in (E-L). (B,C) Staining of centrosomes (γ-tubulin) in control (B) and antisense RNA-expressing cells (C). (M-P) A mitotic cell (transfected with pGRE5/EBV2-BamBgl) where two mitotic poles with much reduced asters have formed and started to separate within a 'cocoon' of chromosomes. (M) The complete image; (N-P) 2 μm optical sections at the bottom, middle and top of the image, respectively. Bar, 25 μm.

released by Ral at the beginning of mitosis. At low concentration levels, it would associate preferentially with its high affinity target in the spindle poles, while when overexpressed there would be enough left free to interfere with microtubule dynamics. The much reduced size of the centrosomes in cytocentrin-overexpressing mitotic cells (Fig. 4B) offers an alternative explanation, suggesting that excess free cytocentrin may have prevented accumulation at the centrosomes of component(s) required for nucleation of microtubules.

Our model implies that, under normal conditions, cytocentrin must be sequestered away from the centrosome until mitosis, raising the question why its over-expression did not precipitate the events which are usually driven by this protein on mitotic centrosomes.

A likely explanation is that such events do not depend on cytocentrin alone, and other centrosomal components, some of which may also be recruited around the same time but under different cellular regulation, are required. Alternatively, resident centrosomal components may have to undergo modifications, such as phosphorylation, in order for cytocentrin to drive the aforementioned events, and it is well known that extensive phosphorylation takes place in the centrosome at the time of mitosis. It is also possible that cytocentrin overexpression may in fact have caused at least some of these events to occur, but in an untimely or unphysiological way, as indicated by the presence of more than two centrosomes in most mitotic overexpressing cells, such as that shown in Fig. 4A-C.

The antisense experiments have shed light into the function of cytocentrin once it is bound to the mitotic poles by demonstrating that, when its concentration is markedly reduced, the poles cannot separate in a timely fashion. This led to the formation of monopolar structures (Fig. 7) reminiscent of those described for two mitotic mutants of *Drosophila*: aurora (*aur*) (Glover et al., 1995) and 'merry-go-round' (*mgr*) (Gonzales et al., 1988). In both cases, the monoastral spindles were described as having about twice the size of the poles of normal bipolar spindles, suggesting that the centrosomes had duplicated but failed to separate. These structures are clearly different from the monopolar spindles extensively investigated by Mazia et al. (1981) and found in cells, with relative frequency depending on animal species, whenever nuclear envelope breakdown precedes separation of the diplosomes. These normally occurring monopolar structures can be best described as half-spindles, with the chromosomes distributed radially on one side of the single pole (Cassimeris et al., 1994). Such an organization is quite different from the spherical arrangement we have observed in COS-1 cells transfected with antisense cytocentrin cDNA (Fig. 7). Our observations are also consistent with partial rather than complete inhibition of mitotic functions and cell cycle progression in cells displaying monopolar spindles. The most likely explanation is that, in the absence of sufficient levels of cytocentrin, separation of the diplosomes was greatly delayed, leading to the formation of spherical monopolar structures containing at their center an enlarged, already duplicated spindle pole. Consistent with such a conclusion is the observation that the stably transfected clones could grow, although at a much reduced rate and with progressive accumulation of abnormalities such as grossly enlarged and hyperdiploid nuclei and massive multipolar mitoses.

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